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RESEARCH ARTICLE | *Translational Physiology*

Parenterial iron sucrose-induced renal preconditioning: differential ferritin heavy and light chain expression in plasma, urine, and internal organs

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Johnson AC, Gooley T, Guillem A, Keyser J, Rasmussen H, Singh B, Zager RA. Parenterial iron sucrose-induced renal preconditioning: differential ferritin heavy and light chain expression in plasma, urine, and internal organs. *Am J Physiol Renal Physiol* 317: F1563–F1571, 2019. First published October 14, 2019; doi:10.1152/ajprenal.00307.2019.—Experimental data suggest that iron sucrose (FeS) injection, used either alone or in combination with other prooxidants, can induce "renal preconditioning," in part by upregulating cytoprotective ferritin levels. However, the rapidity, degree, composition (heavy vs. light chain), and renal ferritin changes after FeS administration in humans remain to be defined. To address these issues, healthy human volunteers ($n = 9$) and patients with stage 3–4 chronic kidney disease($n = 9$) were injected once with FeS (120, 240, or 360 mg). Plasma ferritin was measured from 0 to 8 days postinjection as an overall index of ferritin generation. Urinary ferritin served as a "biomarker" of renal ferritin production. FeS induced rapid (≤2 h), dose-dependent, plasma ferritin increases in all study participants, peaking at approximately three to five times baseline within 24–48 h. Significant urinary ferritin increases (~3 times), without dose-dependent increases in albuminuria, neutrophil gelatinase-associated lipocalin, or *N*-acetyl-β-D-glucosaminidase excretion, were observed. Western blot analysis with ferritin heavy chain (Fhc)- and light chain (Flc)-specific antibodies demonstrated that FeS raised plasma Flc but not Fhc levels. Conversely, FeS increased both Fhc and Flc in urine. To assess sites of FeS-induced ferritin generation, organs from FeS-treated mice were probed for Fhc, Flc, and their mRNAs. FeS predominantly raised hepatic Flc. Conversely, marked Fhc and Flc elevations developed in the kidney and spleen. No cardiopulmonary ferritin increases occurred. Ferritin mRNAs remained unchanged throughout, implying posttranscriptional ferritin production. We conclude that FeS induces rapid, dramatic, and differential Fhc and Flc upregulation in organs. Renal Fhc and Flc increases, in the absence of nephrotoxicity, suggest potential FeS utility as a clinical renal "preconditioning" agent.

acute kidney injury; ferritin heavy chain; ferritin light chain; preconditioning

INTRODUCTION

Over 250,000 patients undergo "on pump" heart surgeries per year (9). Approximately 50% of these cases are coronary artery bypass grafts, with the remaining ~40% being aortic or mitral valve replacements or repair (9). Acute kidney injury (AKI) develops in up to 30% of these patients, with ~1–5% requiring renal replacement therapy (28). Cardiovascular surgery-induced AKI is a serious adverse event, leading to increased hospital length of stay, postoperative complications (27, 32), and the potential onset of progressive renal disease (2, 11, 21). Renal replacement therapy, if required, portends a ~50% mortality rate (14, 25).

Given these considerations, there has been a major interest in developing pharmacological agents to either prevent cardiovascular surgery-induced AKI or attenuate its severity. The former approach is more promising, given that it is easier to prevent AKI than to reverse it once injury has occurred. A potentially useful approach for AKI prevention is so-called "renal preconditioning." With this strategy, a mild renal "stressor" is administered, which triggers the upregulation of diverse renal cytoprotective proteins over the ensuing 24 h (e.g., Refs. 5, 33, and 36–40). As a result, renal resistance to diverse forms of AKI emerges (33, 36, 37). Although it has been recognized for >100 yr that mild renal stressors, e.g., nephrotoxin exposure, can evoke experimental renal "preconditioning" (13), a safe and effective way to clinically recapitulate this state has not yet emerged.

Toward this goal, our laboratory has explored the concept of so-called "oxidant preconditioning" in several experimental studies (15–17, 36–40). The strategy has been to activate redox-sensitive signaling pathways within the kidney by administering prooxidant agents, most notably intravenous iron [iron sucrose (FeS)] given either with or without a heme oxygenase inhibitor (tin protoporphyrin) (36–40). With the induction of mild, transient, oxidant stress, the redox-sensitive nuclear factor erythroid 2-related factor 2 (Nrf2) cytoprotective pathway is activated, causing marked upregulation of diverse antioxidant/cytoprotective proteins (16). Over the ensuing 24 h, striking protection against diverse forms of experimental AKI emerges.

Ferritin upregulation is a critical component of this oxidant preconditioning state (15, 26, 31). This 450,000-Da macromolecule is typically made up of 12 "light" chains (Flc) and 12 "heavy" chains (Fhc). However, the relative proportion of Fhc versus Flc within the ferritin molecule can vary substantially depending on the organ, cell type, and nature of the inducing stimulus (6, 12, 29). The cytoprotective properties of ferritin stem from at least four actions. First, intact ferritin can sequester, and hence neutralize, large amounts of "catalytic" (prooxidant) Fe as it is released from intracellular heme proteins (mitochondria and cytochromes) during evolving cell injury (46). Second, Fhc possesses ferroxidase activity, which con-

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verts prooxidant Fe^{2+} to its less reactive Fe^{3+} form (46). Third, because catalytic Fe can activate an inflammatory cascade (41, 43), ferritin-induced Fe binding exerts an anti-inflammatory effect. Fourth, it has recently been posited that ferritin can exert a variety of protective actions that are independent of iron sequestration (8, 23, 47). Support for the clinical relevance of ferritin's protective actions comes from two recent studies that reported that *1*) pre- or perioperative plasma ferritin levels inversely correlate with the frequency of cardiovascular surgery-induced AKI (7) and *2*) elevated plasma ferritin levels are associated with improved clinical renal transplant outcomes (30).

We have previously documented that FeS has utility as an experimental oxidant preconditioning agent, in part by upregulating ferritin expression (15–17, 36–40). However, translation of these prior experimental findings to the clinical arena rests on several important assumptions. First, FeS must be able to rapidly (e.g., with 12–24 h) and robustly increase ferritin levels in humans if it is to confer rapid protection against impending renal insults, e.g., cardiac surgery. Although FeS is well known to increase ferritin levels in humans, this has classically been documented within weeks, not hours, of its administration (4, 19). Second, it is unknown whether FeS predominantly upregulates Fhc versus Flc expression. Given that the protective actions of ferritin are in large part Fhc dependent (46), FeS-mediated Fhc upregulation seems essential if it is to induce a protective effect. Third, although the liver is the dominant site of ferritin production, its large size (450,000 Da) precludes glomerular filtration, thereby preventing direct tubular access. Thus, if FeS is to induce renal cytoprotection in humans, an increase in intrarenal ferritin/Fhc production would seemingly be required. Finally, FeS "preconditioning" must be well tolerated by the human kidney despite its inducing transient renal oxidant stress (15–17, 36–40). Were FeS to exert overt nephrotoxicity, this would preclude its clinical acceptance as a preconditioning agent. Hence, to gain insights into each of these issues, the present clinical study, complemented with a few supporting mouse experiments, was undertaken.

METHODS

Clinical Experiments

Nine healthy volunteers (HVs) and nine patients with stage 3–4 chronic kidney disease (CKD) were recruited for this investigation. The study received Institutional Review Board (IRB) approval from Advarra IRB (Columbia, MD), and informed consent was obtained from each participant. IRB approval from Fred Hutchinson Cancer Center was waived because only laboratory analysis of deidentified samples was performed at this site. Study exclusion criteria included pregnancy, any significant medical illness other than the presence of CKD, iron administration in the prior 30 days, or a plasma ferritin concentration of >500 ng/mL. Specific demographic data, screening estimated glomerular filtration rate [eGFR; Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation], blood urea nitrogen (BUN), serum creatinine, and blood pressure for the participants are shown in Table 1. This study was undertaken as part of a larger study that was enrolled in Clinicaltrials.gov (NCT03630029). The specific aspects of this subtrial were also Clinicaltrials.gov enrolled (NCT04072432).

The HV and CKD groups were each divided into three equal cohorts ($n = 3$ each). Each cohort received either 120, 240, or 360 mg FeS (12 mg/mL stock solution, Cascade Custom Chemistry, Portland, OR). The FeS dose (10, 20, or 30 mL of stock solution) was infused intravenously with 100 mL saline over 1 h. Participants remained overnight at the study site (Riverside Clinical Research, Edgewater, FL) to screen for potential adverse events and to obtain timed blood and urine samples. Blood and urine samples were collected at baseline (0 h) and at 2, 4, 8, 18, 24, 48, 96, and 192 h after FeS infusion. Plasma ferritin concentrations were measured by Halifax Laboratory Services (Daytona Beach, FL) with a Vista 1500 autoanalyzer (K6440-10445136, Siemens). Urine ferritin was measured by ELISA (ELH ferritin, RayBiotech, Norwich, GA). Selected plasma and urine samples were specifically probed for Fhc and Flc by Western blot analysis as described below (*Western Blot Analysis of Flc and Fhc in Human Plasma/Urine and Mouse Tissue Samples*).

Table 1. *Study participant demographics*

	Age	Sex	Weight, kg	Blood Pressure (Sysolic/Diastolic), mmHg	Estimated Glomerular Filtration Rate, mL/min	Blood Urea Nitrogen, mg/dL	Serum Creatinine, mg/dL	Diabetes Mellitus
Dose, 120 mg								
HV	59	Female	64.9	127/88	>70	9.0	0.69	No
HV	51	Male	93.6	130/78	>70	16.0	0.90	No
HV	55	Female	83.0	117/78	>70	12.0	0.90	No
CKD	72	Male	103.0	140/71	29	42.0	2.20	Yes
CKD	59	Female	111.2	121/88	47	22.0	1.26	No
CKD	64	Female	82.5	133/78	34	36	1.47	Yes
Dose, 240 mg								
HV	62	Male	111.5	140/79	>70	11.0	1.04	No
HV	50	Male	100.7	124/84	>70	18.0	1.18	No
HV	31	Female	93.0	110/77	>70	12.0	0.75	No
CKD	66	Female	71.4	154/84	30	26.0	1.74	Yes
CKD	66	Female	101.2	104/72	52	16.0	1.10	No
CKD	71	Female	88.8	146/80	40	20.0	1.54	Yes
Dose, 360 mg								
HV	50	Male	111.2	115/77	>70	12.0	1.17	No
HV	59	Male	73.3	135/79	>70	13.0	1.13	No
HV	51	Male	93.4	127/80	>70	27.0	1.27	No
CKD	74	Male	103.9	125/53	27	37.0	2.28	Yes
CKD	68	Female	79.1	112/72	50	18.0	1.12	No
CKD	78	Female	109.4	136/77	44	12.0	1.19	Yes

Demographics and biological data pertaining to the 9 healthy volunteers (HVs) and 9 patients with chronic kidney disease (CKD) are presented according to the Fe sucrose dose.

Assessments of Potential FeS-Mediated Nephrotoxicity in Humans

To gauge whether FeS evoked nephrotoxicity, three independent, well-established AKI biomarkers were used: *1*) urinary albumin concentrations, reflecting both potential changes in glomerular permselectivity and/or decreased proximal tubule reabsorption (18, 35); *2*) urinary neutrophil gelatinase-associated lipocalin (NGAL), a marker of stress gene upregulation (22, 24); and *3*) urinary *N*-acetyl-β-D-glucosaminidase (NAG), a marker of direct tubular injury as reflected by tubular release of this lysosomal enzyme (10, 22). Albumin was measured with a fluorometric assay (K550-100, BioVision, Milpitas, CA). NGAL was determined by ELISA (DY1857, R&D Systems, Minneapolis, MN), and NAG was quantified by a colorimetric assay (DNAG-100, Bioassay Systems). All values were factored by the urinary creatinine concentration (Z5030020, BioChain, Newark, CA) in each test urine sample. The assessed time points were as follows: baseline and 8, 24, and 48 h after 120, 240, and 360 mg FeS administration. BUN and plasma creatinine concentrations at these three time points were also assessed (Halifax Laboratories).

Mouse Experiments

Organ-specific responses to FeS injection. The following experiment was undertaken to determine *1*) organ sites of FeS-mediated ferritin generation, *2*) whether Fhc versus Flc was produced, and *3*) whether any ferritin upregulation occurred via increased Fhc/Flc gene transcription, as assessed by potential increases in Fhc and Flc mRNAs. Male CD-1 mice (35–40 g, Charles River Laboratories, Wilmington, MA) were used for all animal experiments, which were approved by the institution's Institutional Animal Care and Use Committee. Ten mice were injected via the tail vein with either 1 mg FeS or vehicle ($n = 5$ each). Four hours later, they were deeply anesthetized with pentobarbital (40–50 mg/kg), the abdominal and thoracic cavities were opened through midline incisions, and the heart, lung, kidneys, spleen, and liver were resected. The organs were iced, and total RNA and protein were extracted (15). Renal cortical, hepatic, splenic, cardiac, and lung samples were assayed for Fhc and Flc mRNAs by RT-PCR using the primer pairs shown in Table 2. Values were factored by a simultaneously determined GAPDH product. Fhc and Flc protein levels were probed by Western blot analysis as described below. To determine the durability of the changes observed at 4 h after FeS injection, mice were injected with FeS or vehicle ($n = 3$ each), and 18 h later, Fhc and Flc protein and mRNA assessments in the kidney and liver were made.

Western Blot Analysis of Flc and Fhc in Human Plasma/Urine and Mouse Tissue Samples

To assess the degrees to which Fhc and Flc ferritin are induced by FeS, human plasma and urine and mouse kidney, liver, heart, lung and spleen tissue homogenates were evaluated by Western blot analysis. Normalized, denatured protein, under reduced conditions, was electrophoresed on Criterion XT Precast gels (4–12% bis-Tris, no. 3450124, Bio-Rad, Hercules, CA) and transferred onto 0.45 μm of nitrocellulose using the semidry method. To prevent nonspecific binding, each blot was blocked with 5% nonfat milk before primary antibody incubation. The following Fhc antibodies were used: mouse tissue (ab183781, Abcam, Cambridge, MA) and human plasma or urine (ab75972, Abcam). For Flc detection, the following primary antibodies were used: mouse tissue (ab69090, Abcam) and human plasma or urine (ab243096, Abcam). Goat anti-rabbit secondary antibody was used for all blots (ab97051, Abcam), and binding was assessed by chemiluminescence.

Table 2. *Mouse Primers for RT-PCR*

mRNA	Primer Sequences	Product Size, bp
Heavy chain ferritin		
Forward	5′-CGGTTTCCTGCTTCAACAGTGCTT-3′	741
Reverse	5′-ATGCACTGCCTCAGTGACCAGTAA-3′	
Light chain ferritin		
Forward	5′-GGGCCTACACCTACCTC-3′	265
Reverse	5′-AGATCCAAGAGGGCCTGATT-3′	
GAPDH		
Forward	5′-CTGCCATTTGCAGTGGCAAAGTGG-3′	437
Reverse	5′-TTGTCATGGATGACCTTGGCCAGG-3′	

Primer pairs used for the detection of mouse ferritin heavy chain and ferritin light chain mRNAs by competitive RT-PCR are shown. The GAPDH product served as the housekeeping gene.

Statistics

The primary end points of the clinical portion of this study were change in plasma ferritin levels and change in urine ferritin levels from baseline (before FeS administration) to 24 and 48 h post-FeS administration. These time points were selected before study initiation because this is the timeframe during which the height of the experimental renal preconditioning state is expressed, as previously shown (15–17, 39, 40). Statistical comparisons of change from baseline at these two time points were made using a one-sample *t* test. Fold change was considered to be of primary interest, so the change from baseline was estimated as a ratio. These ratios were then transformed with a logarithm function of base 2, resulting in a difference in logs as our primary outcome. In other words, testing if $\log_2$(ferritin at 24/48 h) – $\log_2$(ferritin at baseline) is 0 is equivalent to testing if the ratio (ferritin at 24 h)/(ferritin at baseline) equals 1. Since the post-FeS ferritin level and the pre-FeS level are paired, a one-sample *t* test was used to test the null hypothesis that the mean of the difference in log-transformed values (post-FeS plasma level − pre-FeS plasma level) is equal to 0 (equivalent to testing if the ratio is equal to 1). If the value of difference in log-transformed values is 1, this corresponds to a doubling of plasma ferritin; if the difference is 2, this corresponds to a 4-fold change; in general, if the difference in log-transformed values is n, this corresponds to a 2^n-fold change. For purposes of interpretability, the difference in the logarithms were exponentiated (with a base of 2) to get a fold change, and the median fold change is presented (the median is used as the summary measure for these purposes as fold changes are inherently nonsymmetric). Sensitivity analyses were conducted to assess the impact of CKD (vs. HV), sex (men vs. women), and FeS dose (120 vs. 240 vs. 360 mg) post-FeS administration on the change from baseline (at 24 and 48 h) using simple linear regression. Secondary analyses were conducted using all post-FeS plasma values and, essentially, asking if the average fold change among these values is different from 1. Since the various ferritin levels within a participant are correlated, we used repeated-measures ANOVA to assess the change from baseline; this allowed us to consider the within-participant variability of ferritin levels. As with change from baseline at 24 and 48 h, change from baseline was modeled as the difference in the log-transformed ferritin values, and this change was regarded as the outcome in the repeated-measures ANOVA. Similar sensitivity analyses as described above were conducted for these secondary analyses in addition to assessing the impact of time post-FeS administration (2, 4, 8, 12, 18, 24, 48, 72, 96, and 192 h) on the change from baseline. Additional secondary analyses assessed the change in urine albumin, urine NAG-to-creatinine ratio, and urine NGAL-to-creatinine ratio from baseline to 24 and 48 h, with these changes being analyzed as detailed above. Other secondary analyses looked at change in plasma ferritin from baseline to the earliest time (2 h) and latest time (192 h) to assess the rapidity of increase from baseline as well as the persistence of the change. Since our primary end point of change in the plasma and urine ferritin level from baseline to 24 and 48 h consisted of four outcomes, in addition to nominal (unadjusted) *P* values we also present Bonferroni-adjusted *P* values accounting for these four comparisons. This adjust-

ment is very conservative, however, as these four outcomes are clearly highly correlated. Only nominal *P* values are provided for secondary analyses as well as selected sensitivity analyses previously described.

RESULTS

Study Participant Baseline Information

Selected demographics and baseline information for each of the study participants are shown in Table 1. Baseline eGFR for the CKD group was 39.2 ± 9.5 mL/min. eGFR for each member of the HV group was reported as >70 mL/min. Baseline serum creatinine levels were 1.54 ± 0.5 and 1.0 ± 0.2 mg/dL for the CKD and HV participants, respectively. Six of nine patients with CKD, but none of the HVs, were classified as having diabetes. Blood pressures were comparable between the HV and CKD groups (mean values: 127/79 mmHg for the HV group and 130/75 mmHg for the CKD group). All participants received the full scheduled FeS dosage. No adverse events that could be ascribed to Fe infusion were noted (1 case of "nasal congestion" and 1 case of "fatigue").

Plasma Ferritin Responses to Intravenous Iron

Individual plasma ferritin levels for each of the participant at each of the assessed time points and with each FeS dose are presented in Supplemental Table S1 (available online at https://doi.org/10.6084/m9.figshare.9779192.v1). Baseline ferritin values varied widely among the participants (means ± SD; HV group: 62 ± 76 ng/mL and CKD group: 99 ± 101 ng/mL). The mean difference from baseline in log-transformed ferritin values is shown by dose in Fig. 1 and by disease (CKD vs. HV) in Fig. 2.

For the primary end points of change in plasma ferritin levels from baseline to 24 and 48 h, the mean change in the log-transformed values was 1.93 (nominal and Bonferroni-adjusted $P < 0.0001$; Table 3). The corresponding median fold increase was 3.35. At 48 h, similar qualitative conclusions were reached, with a mean change in log-transformed values of 2.25 (nominal and Bonferroni-adjusted $P < 0.0001$; Table 3). The corresponding median fold increase was 4.17. All 18 participants had an increase in plasma ferritin level from baseline to 24 and 48 h.

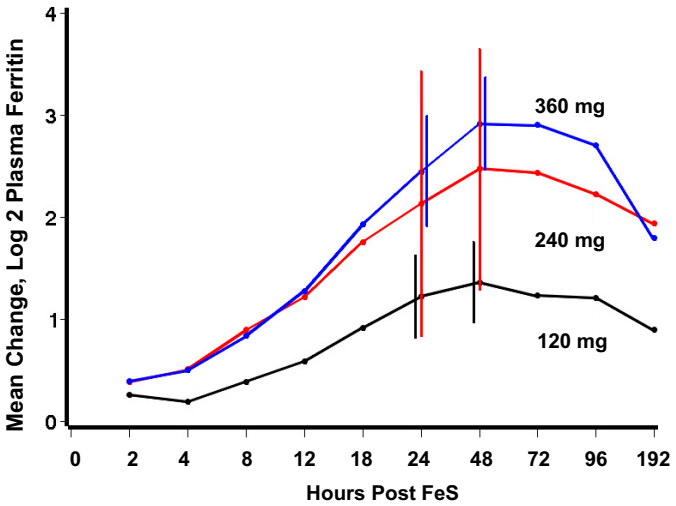

Fig. 1. Change in plasma ferritin concentrations from baseline over time after Fe sucrose (FeS) administration by dose (120, 240, and 360 mg) for all study participants combined. The data represent the mean change from baseline in log-transformed values. Individual vales for each participant and at each time point are provided in Supplemental Table S1 (available online at https://doi.org/10.6084/m9.figshare.9779192.v1). The 95% confidence intervals at the primary end points, 24 and 48 h post-FeS, are shown.

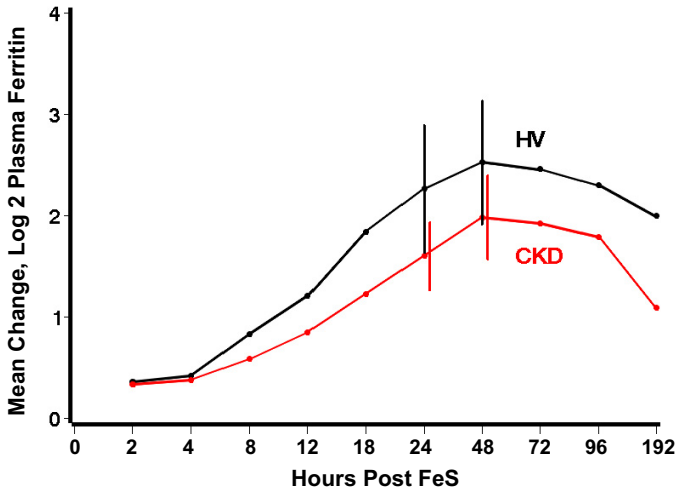

Fig. 2. Changes in plasma ferritin concentrations after Fe sucrose (FeS) administration for health volunteers (HVs) versus patients with chronic kidney disease (CKD). The data represent the mean difference from baseline in log-transformed values. Individual vales for each participant and at each time point are provided in Supplemental Table S1 (available online at https://doi.org/10.6084/m9.figshare.9779192.v1). The 95% confidence intervals at the primary end points, 24 and 48 h post-FeS, are shown.

Because each participant had an increase from baseline to both 24 and 48 h, sensitivity analyses showed an increase, on average, from baseline in every subset of participants based on the presence of CKD, sex, and dose of FeS. In particular, the mean change in log-transformed values at 24 h was 1.98 in women and 1.87 in men, respectively; the mean change was 2.26 in HVs and 1.60 in patients with CKD, respectively. Results were qualitatively similar for change from baseline to 48 h, with a mean change of 2.31 in women, 2.18 in men, 2.52 in HVs, and 1.98 in patients with CKD. Further sensitivity analyses suggested a dose-dependent effect, as the mean change in log-transformed values from baseline to 24 h was 1.22, 2.13, and 2.45 for doses of 120, 240, and 360 mg, respectively. For the change at 48 h, the mean changes were 1.36, 2.47, and 2.92, respectively.

Secondary analyses that assessed change from baseline at all times, not just at 24 and 48 h, yielded qualitatively similar results. Among all participants (HVs and patients with CKD at all doses), there were 180 plasma ferritin values at all time points after FeS administration. Of these, 178 values were increased compared with the corresponding baseline value, and the mean change in log-transformed values was 1.40 ($P < 0.0001$ by repeated-measures ANOVA), with a range of −1.35 to 5.29. The increases in ferritin levels from baseline did not show obvious differences between HVs and patients with CKD ($P = 0.23$) or based on sex ($P = 0.79$). There was little suggestion of a statistical interaction between sex and presence of CKD ($P = 0.79$), although the number of participants precluded a reasonable assessment of any interactions ($n = 2$ men with CKD, $n = 6$ male HVs, $n = 7$ women with CKD, and $n = 3$ female HVs). The ferritin response increased as the FeS

Table 3. *Changes in plasma and urine ferritin values from baseline to 24 and 48 h after Fe sucrose administration*

	Plasma Ferritin, ng/mg creatinine		Urine Ferritin, ng/mg creatinine	
	24 h	48 h	24 h	48 h
Means (SD) change from baseline in log-transformed values	1.93 (1.12) ($P < 0.0001$, $P < 0.0001$)	2.25 (1.12) ($P < 0.0001$, $P < 0.0001$)	2.22 (1.93) ($P = 0.0001$, $P = 0.0006$)	1.74 (2.33) ($P = 0.006$, $P = 0.02$)
Median fold change from baseline	3.35	4.17	3.93	2.62

Mean values of difference in the $\log_2$-transformed values are shown (P values from a one-sample t test, unadjusted and Bonferroni adjusted). For ease of interpretation, the median fold change from baseline is also shown.

dose increased (mean difference in log-transformed values of 0.82, 1.60, and 1.77 for doses of 120, 240, 360 mg, respectively, $P = 0.03$).

An increase, albeit modest, in plasma ferritin was seen at the earliest time point of 2 h. The mean change in log-transformed values was 0.34 ($P < 0.0001$, corresponding median 1.25-fold increase). Of note, each of the 18 patients had an increase from baseline to 2 h. The mean change at 2 h was similar in HV and CKD groups (0.36 and 0.33, respectively). At 192 h, the mean change in log-transformed values was 1.54 ($P < 0.0001$, corresponding median 2.76-fold increase). Of note, 17 of 18 patients had a higher plasma ferritin level at 192 h than at baseline. The mean increase at 192 h was 2.00 in the HV group and 1.08 in the CKD group.

Western Blot Analysis of Human Plasma Ferritin

It is noteworthy that clinically deployed ferritin auto-analyzer assays do not provide information as to the relative amounts of heavy chain versus light chain that are present. Thus, to gain specific information in this regard, baseline plasma samples and urine samples obtained 24–48 h after 240–360 mg Fe infusion were probed by Western blot analysis using either heavy chain- or light chain-specific antibodies. As shown in Fig. 3, *lanes A* and *B*, FeS markedly increased plasma light chains in the absence of discernible heavy chain increases. To determine whether the observed light chain increases reflected free light chains within the circulation (i.e., not incorporated into the intact 450-kDa ferritin molecule), 48-h post-FeS plasma samples were filtered through a 50-kDa molecular mass exclusion filter and the recovered (low molecular mass) fractions were assayed by ferritin ELISA. No ferritin was detected, implying that the plasma light chain increases were not free within the circulation. The retained fraction contained ferritin concentrations that approximated the auto-analyzer results (data not shown).

Human Urine Ferritin

Because of its large size (450 kDa) and the absence of potentially filterable free Flc or Fhc within the circulation (as determined above), the presence of ferritin in urine is presumed to reflect a renal origin (20). Hence, we measured urinary ferritin and expressed the results as nanograms per milligrams of creatinine. At baseline, patients with CKD had higher urine ferritin levels than did HVs (32.88 vs. 2.26 ng/mg). At 24 h after FeS injection, the mean difference in log-transformed urine ferritin values was 2.22 (nominal $P = 0.0001$, Bonferroni-adjusted $P = 0.0006$; Table 3) with a median fold change of 3.93. There were mean increases in log-transformed values in both HV (2.46) and CKD (1.97) groups. Of note, all 18 patients had an increase from baseline in urine ferritin at 24 h. Similar results were seen at 48 h, with a mean difference in log-transformed values of 1.74 (nominal $P = 0.006$, Bonferroni-adjusted $P = 0.02$; Table 3; mean differences of 1.51 and 1.92 in HV and CKD groups, respectively) and a median fold change of 2.62. Seventeen of eighteen subjects had an increase from baseline to 192 h. The mean change from baseline in log-transformed values depicted by dose is shown in Fig. 4. Absolute urine ferritin/creatinine values at baseline and 24 and 48 h post-FeS injection are shown in

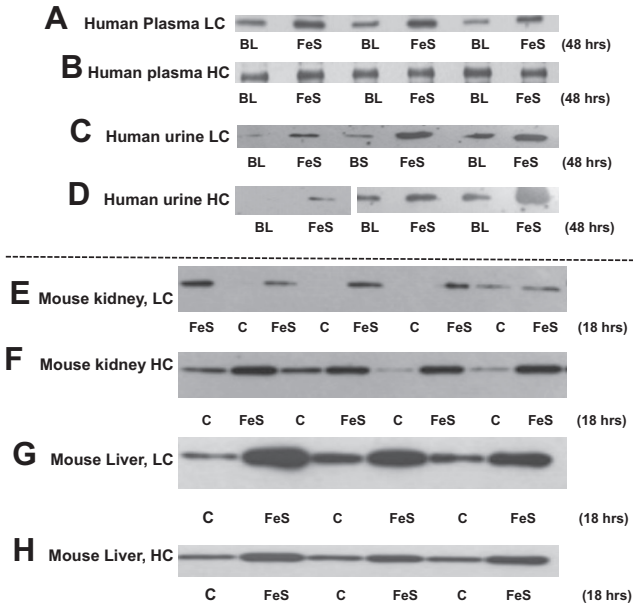

Fig. 3. Western blots of human plasma and urine as well as mouse kidney and liver samples using ferritin heavy chain (HC)- and ferritin light chain (LC)-specific antibodies. Lane samples, representative of overall Western blot probes, are shown. As depicted, at 48 h after 240 mg Fe sucrose (FeS) administration, human plasma manifested a marked increase in LC ferritin (*lane A*) but not HC ferritin (*lane B*) compared with baseline (BL) levels. Of note, the maximal increase in plasma ferritin, as detected by ELISA, was at 48 h; thus, this time was selected for Western blot analysis. Human urine showed both LC (*lane C*) and HC (*lane D*) responses to FeS treatment. (Samples from both patients with chronic kidney disease and healthy volunteers revealed comparable changes; not shown.) In *lanes E–H*, mouse kidney and liver blots are shown at 18 h post-FeS administration and compared with blots obtained using control (C) kidney and liver tissues. First, the mouse kidney showed virtually no LC expression in the absence of FeS treatment; however, by 18 h post-FeS, marked renal LC accumulation was observed (*lane E*). Second, the mouse kidney revealed HC expression under control conditions, and marked HC increases resulted from FeS treatment (*lane F*). Third, in the liver, both HCs and LCs were expressed under control conditions (*lanes G* and *F*). FeS most prominently upregulated hepatic LC expression.

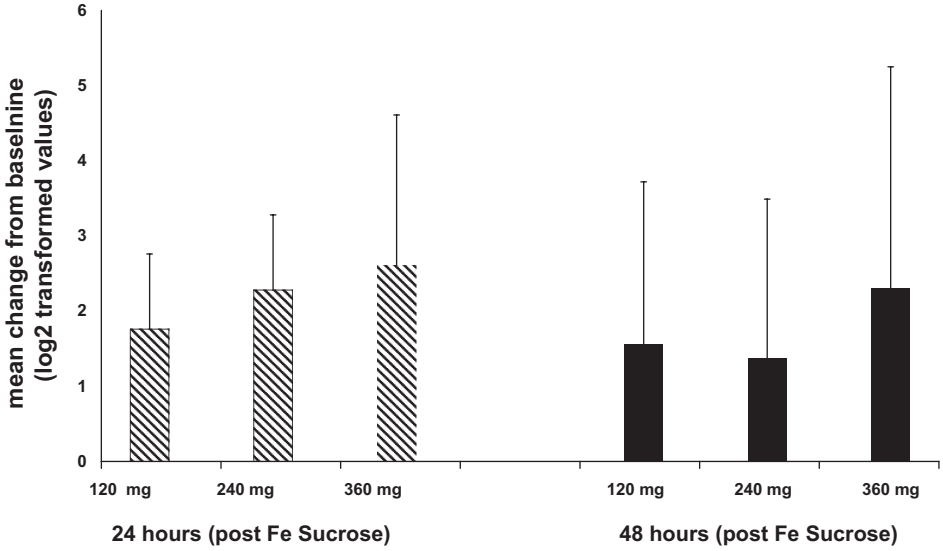

Fig. 4. Change in urine ferritin concentrations (as assessed by ELISA) from baseline to 24 and 48 h after Fe sucrose administration by dose (120, 240, and 360 mg). The bar heights represent the mean change from baseline in log-transformed values. Individual urine ferritin values, factored by urine creatinine at baseline and at 24 and 48 h postiron administration, are provided in Supplemental Table S2 (available online at https://doi.org/10.6084/m9.figshare.9779192.v1). The vertical lines represent the 95% confidence limits.

Supplemental Table S2 (available online at https://doi.org/10.6084/m9.figshare.9779192.v1).

Human Urine Ferritin Western Blot Analysis

The above noted increase in urinary ferritin, as determined by ELISA, reflected increases in both light and heavy chains (see Fig. 3, *lanes C* and *D*). This stands in contrast to the previously noted plasma Western blot analyses that demonstrated only a light chain increase in response to FeS injection.

Assessment of Potential FeS Nephrotoxicity in Humans

Urinary albumin concentrations. Baseline urine albumin-to-creatinine ratios were higher in the CKD group versus the HV group (0.73 vs. 0.11 mg/gm creatinine, respectively). Consistent with the findings of Agarwal et al. (1), some (10 of 18) participants manifested minor increases in urine albumin concentrations within 8 h of Fe administration (Supplemental Table S3, available online at https://doi.org/10.6084/m9.figshare.9779192.v1). These increases occurred even less frequently at 24 and 48 h; the mean change in log-transformed values was −0.08 (increase in 9 of 18 participants) and −0.31 (increase in 6 of 18 participants), respectively. There was no suggestion of a dose-response association with the mean change in log-transformed values from baseline to 24 h (means of 0.42, −0.64, and −0.03 for 120, 240, and 360 mg, respectively) or 48 h (0.02, −0.42, and −0.52, respectively), implying a possible infusion effect rather than direct FeS toxicity.

Urinary NAG concentrations. Baseline urinary NAG-to-creatinine values were approximately twice as high in the CKD group (4.66) versus the HV group (2.26). There were modest increases from baseline to 24 h (12 of 18 participants) and 48 h (11 of 18 participants), with a mean change in log-transformed values of 0.45 ($P = 0.004$) and 0.32 ($P = 0.02$), respectively. These increases were similar in HVs and patients with CKD (0.45 vs. 0.44, respectively, at 24 h and 0.30 vs. 0.33, respectively, at 48 h; Supplemental Table S4, available online at https://doi.org/10.6084/m9.figshare.9779192.v1). There was no suggestion of a dose-response association with the mean change in log-transformed values to 24 h (mean changes of 0.47, 0.49, and 0.38 for 120, 240, and 360 mg, respectively) or 48 h (0.23, 0.48, 0.24), again suggesting a possible infusion effect rather than direct FeS toxicity.

Urinary NGAL concentrations. Individual urinary NGAL-to-creatinine ratios are provided in Supplemental Table S5 (available online at https://doi.org/10.6084/m9.figshare.9779192.v1). Baseline urinary values were quite variable but were, on average, higher in patients with CKD compared with HVs (110.9 vs. 10.1 ng/mg). The changes from baseline were quite variable; there were some increases from baseline to 24 h (10 of 18 participants) and to 48 h (7 of 18 participants), with a mean change in log-transformed values of 0.63 and 0.09, respectively), but these increases were not convincingly real and/or were, at most, modest ($P = 0.25$ and $P = 0.80$, respectively). The mean changes in log-transformed values in HVs and patients with CKD were 0.88 and 0.39, respectively, at 24 h and −0.41 vs. 0.59, respectively, at 48 h. There was no suggestion of a dose-response association with change from baseline to 24 h (mean change in log-transformed values of 0.38, 1.22, and 0.30 for 120, 240, and 360 mg, respectively) or 48 h (−0.06, 0.43, and −0.10).

Mouse Experiments: Probing Mouse Organs for Heavy and Light Chains

The kidney. As discussed above, it was assumed that the increase in urine Fhc and Flc seen in HVs and patients with CKD reflected renal production rather than ferritin filtration due to ferritin's large size (450 kDa). However, to provide experimental proof that the kidney produces both Fhc and Flc, we probed mouse renal cortical extracts at 4 and 18 h post-FeS administration. At baseline, Fhc, but not Flc, Western blot bands were observed (Fig. 3). After FeS administration, marked Flc and Fhc increases were noted at both the 4- and 18-h time points. Representative baseline plots and 18-h blots are shown in Fig. 3, *lanes E* and *F*.

Extrarenal organs. Baseline liver samples demonstrated both Flc and Fhc expression. FeS induced marked Flc, but only small Fhc, hepatic increases (Fig. 3, *lanes I* and *H*). The spleen demonstrated substantial Fe-induced Flc and Fhc increases (not shown), recapitulating the kidney pattern noted above. FeS failed to alter either Flc or Fhc in the heart or lung (not shown).

Mouse Ferritin mRNA Assessments

FeS induced no significant increases in Flc or Fhc mRNA in any of the tested organs (Table 4), implying that translational, rather than transcriptional, events were responsible for the above noted FeS-mediated ferritin increases.

DISCUSSION

A classic experimental maneuver for upregulating ferritin, and thereby triggering its cytoprotective properties, is the administration of heme proteins (most notably hemoglobin or myoglobin; e.g., Refs. 26, 31, and 36). Within 24 h of their injection, striking increases in renal ferritin levels, and renal resistance to AKI, result. However, given the propensity of heme proteins to coprecipitate with Tamm-Horsfall protein (i.e., uromodulin) in the distal nephron under aciduric conditions (34), the potential for obstructive cast formation precludes heme protein use as clinical preconditioning agents. Given this consideration, our laboratory previously tested whether FeS can recapitulate heme protein-mediated ferritin upregulation and thus have potential utility as a renal protective agent (15–17, 36–40). Our prior experimental results strongly support this possibility, given that within 18–24 h of FeS administration, significant ferritin upregulation and partial protection against diverse forms of AKI develop (15–17, 36–40).

For FeS to have clinical utility as a renal preconditioning agent, a rapid ferritin response (e.g., within 12–24 h before cardiovascular surgery), including an increase in renal ferritin levels, is presumably required. Hence, the present clinical study was undertaken to assess the time course of FeS-induced ferritin upregulation using plasma and urine ferritin levels as biomarkers of this response. Surprisingly, within just 2 h of FeS administration to either HVs or patients with CKD, significant, dose-dependent, plasma ferritin elevations were observed. These increases were progressive with time, rising approximately three- to fivefold above baseline values in both the HV and CKD cohorts within just 24–48 h. It is notable that the plasma ferritin increases were well maintained throughout the 8-day study period. Thus, the above data suggest that the FeS-mediated ferritin response is sufficiently rapid, robust, and sustained to have potential utility as a clinical preoperative renal preconditioning agent.

Although we have previously demonstrated that FeS induces a marked increase in renal ferritin levels in mice (15–17, 36–40), we had no evidence that the same holds true in humans. Thus, to gain insight into this matter, we used urinary ferritin concentrations as a biomarker of human kidney ferritin expression (20). By 24–48 h post-FeS administration, approximately three- to fivefold urinary ferritin elevations were observed. Three factors support the concept that these urinary ferritin increases do, indeed, reflect renal ferritin induction. First, given its large size (450 kDa), circulating ferritin is far too large to pass through the glomerular filter, particularly in HVs with an intact glomerular barrier. This implies that increased renal ferritin production, with partial ferritin egress into tubular lumina, occurs. Second, if one were to contend that FeS damages the glomerulus, thereby facilitating ferritin filtration, one would expect a corresponding (and even larger) increase in urinary albumin excretion given its much smaller size (69 kDa). However, no matching urinary albumin increases were observed. This precludes increased glomerular filtration as the reason for increased urinary ferritin excretion. Third, we considered the possibility that the urinary ferritin increases could theoretically result from the filtration of low-molecular-mass free heavy or free light chains (23 and 21 kDa, respectively) into urine. However, when we filtered postiron-treated human plasma samples through a 50-kDa exclusion filter, no low-molecular-mass ferritin moieties were detected in the filtrate. Thus, these three findings support the concept that the detected urine ferritin increases after FeS treatment reflected increased renal ferritin production. Of interest, it has previously been documented that clinical hemoglobinuria also increases urinary ferritin excretion [so-called "ferritinuria" (20)]. This further implies that an upregulation of renal ferritin expression is marked by increased urinary ferritin excretion. Of note, the post-Fe urinary ferritin levels varied substantially among tested study participants. Whether relatively high versus low urinary ferritin responders might predict degrees of renal protection is an intriguing consideration.

Table 4. *Ferritin heavy and light chain mRNA levels in mouse test organs either under control conditions or 4 h post-FeS injection*

	Fhc mRNA (4 h)	Flc mRNA (4 h)	Fhc mRNA (18 h)	Flc mRNA (18 h)
Kidney				
FeS	1.49 ± 0.06	1.47 ± 0.09	1.40 ± 07	1.35 ± 0.17
Control	1.38 ± 0.01	1.10 ± 0.09	1.39 ± 0.09	1.41 ± 0.17
Liver				
FeS	1.71 ± 0.14	3.63 ± 0.13	1.31 ± 0.08	2.32 ± 0.21
Control	1.77 ± 0.11	3.65 ± 0.15	1.75 ± 0.12	2.8 ± 0.17
Heart				
FeS	0.58 ± 0.03	0.09 ± 0.01 (6:1)	ND	ND
Control	0.55 ± 0.04	0.09 ± 0.01	ND	ND
Lung				
FeS	1.80 ± 0.0.05	1.53 ± 0.1	ND	ND
Control	1.72 ± 0.12	1.82 ± 0.12	ND	ND
Spleen				
FeS	2.99 ± 0.51	1.97 ± 0.23	ND	ND
Control	2.72 ± 0.68	1.87 ± 0.22	ND	ND

All organs were tested at 4 h postinjection (($n = 5$ each). Kidney and liver mRNAs were also assessed at 18 h postinjection ($n = 3$ each). Differences between Fe sucrose (FeS)-injected mice and control mice were similar. Fhc, ferritin heavy chain; Flc, ferritin light chain; ND, not determined.

When interpreting plasma or urine ferritin ELISA results, it is important to recognize that clinically deployed ferritin assays are neither Fhc nor Flc specific. This is because there is ~50% homology between the two ferritin chains and ELISA-based assays do not distinguish between them. Depending on the type of tissue assayed, the ratio of heavy chains to light chains can substantially differ (e.g., in the liver and spleen: Flc >> Fhc and in the kidney and heart: Fhc > Flc) (6, 12, 29). Furthermore, the relative proportions of Flc and Fhc can vary under different physiological/pathophysiological conditions (12). Given these considerations, and given that FeS evoked marked plasma and urine ferritin increases as assessed by ELISA, we sought to determine whether FeS preferentially evoked Fhc versus Flc expression. To this end, we performed Western blot analysis of clinical plasma and urine samples using Fhc- and Flc-specific antibodies that were raised against synthetic non-homologous Fhc and Flc amino acid sequences. FeS evoked dramatic plasma Flc, but not Fhc, increases. Conversely, comparable urinary Fhc and Flc increases were observed. To gain more specific insights into organ-specific patterns of Fhc versus Flc production in response to FeS, we probed mouse organ samples. The liver manifested a marked Flc, but a lesser Fhc, response to FeS injection, consistent with the pattern observed in human plasma. Conversely, comparable Fhc and Flc increases were seen in the kidney, again consistent with the human urine pattern. Of note, the spleen recapitulated these renal results, whereas neither the heart nor lung manifested FeS-induced ferritin increments. In concert, these findings indicate that different organs demonstrate different ferritin responses to FeS and that the kidney and spleen are the dominant (tested) organs to mount Fhc increases. Finally, it is well established that both transcription and translation can increase ferritin production (29). That FeS failed to substantially increase either Fhc or Flc mRNA in any of the tested organ suggests that FeS predominantly stimulated ferritin production via translational rather than transcriptional events.

For FeS to be a clinically acceptable renal preconditioning agent, it must induce this response in the absence of a significant nephrotoxic effect. In this regard, we have previously reported that when FeS was administered to mice in suprapharmacological doses, mild tubular toxicity was, indeed, observed (42, 44). Thus, we have now explored whether the currently used FeS doses (120, 240, and 360 mg) would be well tolerated by both normal and diseased (stage 3–4 CKD) human kidneys. This appears to be the case, given that BUN and creatinine levels remained stable post-FeS treatment and that no significant dose-dependent increases in urinary NAG, NGAL, or albumin concentrations were observed. Of note, all three of these urinary biomarkers appeared elevated at baseline in the CKD versus HV cohorts, underscoring their role as renal injury biomarkers.

The ultimate clinical utility of FeS-induced preconditioning remains to be defined, and the present study has a number of limitations in this regard. First, although it is clear that ferritin possesses potent cytoprotective properties, the degree to which they are expressed is presumably dependent on cellular, not plasma or urinary, ferritin concentrations. In this regard, it remains to be determined how accurately plasma and urine ferritin assays reflect intracellular ferritin pools. Second, while the present mouse results clearly indicate that FeS increased renal Fhc and Flc levels, only direct human tissue ferritin analysis, currently not feasible, can provide definitive data. Third, although FeS can clearly evoke oxidant preconditioning, it should be emphasized that many other redox-sensitive proteins in addition to ferritin contribute to the emergence of a postoxidant renal cytoprotective state. Notable in this regard are heme oxygenase-1, haptoglobin, and hemopexin, which can also be upregulated by Fe-mediated preconditioning (3, 15–17, 36–40, 45). Thus, the potential efficacy of FeS as a preconditioning agent cannot be gauged solely by ferritin assessments. Finally, in our prior experimental work, we have documented that the coadministration of a transient heme oxygenase inhibitor, tin protoporphyrin, dramatically enhances the FeS-induced preconditioning state (15–17, 39, 40). Hence, combination agent administration is likely to have greater potential for inducing clinical success. Each of the above issues will obviously necessitate further clinical studies, some of which are currently in the planning stage.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

T.G., A.G., J.K., H.R., B.S., and R.A.Z. conceived and designed research; A.C.J. performed experiments; A.C.J., T.G., A.G., J.K., H.R., B.S., and R.A.Z. analyzed data; T.G., A.G., J.K., B.S., and R.A.Z. interpreted results of experiments; T.G., A.G., J.K., H.R., B.S., and R.A.Z. prepared figures; A.G., J.K., B.S., and R.A.Z. drafted manuscript; A.C.J., T.G., A.G., J.K., H.R., B.S., and R.A.Z. edited and revised manuscript; A.C.J., T.G., A.G., J.K., H.R., B.S., and R.A.Z. approved final version of manuscript.

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